Differentiating Modes of Drug Induced Liver Injury Using Parallel Reaction Monitoring LC-MS

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Overview

Introduction

Determining the hepatic safety risk of new therapeutic molecules is an essential aspect of early clinical drug development. Currently, liver function is evaluated in the clinic by measuring levels of a few select markers including aminotransferases, ALT and AST, and bilirubin in the blood.

In contrast to clinical lab chemistries, LC-MS methodologies enable efficient quantitation of large numbers of analytes from small volumes. By expanding the current liver injury biomarker panel and increasing the number of sampling time points via in-home collection using dried blood spots (DBS) or similar microsampling techniques such as volumetric absorptive microsampling (VAMS), richer datasets may be generated. This could improve our understanding of different mechanisms of drug induced liver injury (DILI) and help to guide drug development.

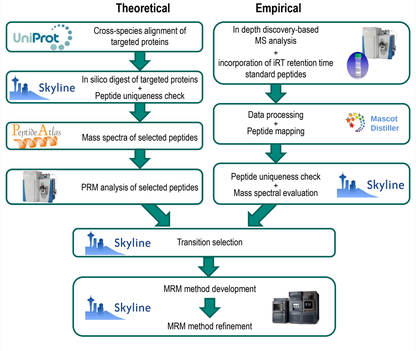
Approach

1. Based on previous study and literature, a panel of potential liver injury biomarker proteins was targeted for method development
2. Skyline library containing the MS/MS spectra and retention time information was constructed for targeted proteomic analysis (**Figure 1**)
3. iRT was calculated and incorporated for target peptides to enable scheduled parallel reaction monitoring (PRM)
4. PRM was applied to quantify protein fold changes in rat plasma following treatment with drug or vehicle control
5. Feasibility of DILI biomarker quantitation from DBS and VAMS was assessed

Methods

Assay Development

Figure 1. Skyline library construction

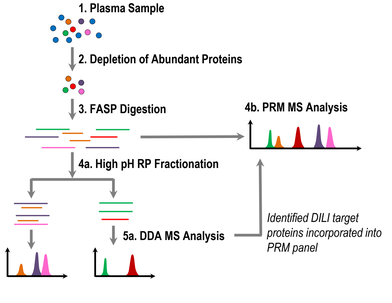


Abundant protein depletion was performed with Agilent Multiple Affinity Removal Spin Cartridges (MARS).

Filter-aided sample preparation (FASP) was used for reduction, alkylation, and digestion.

Resulting peptides were fractionated with high pH reversed-phase HPLC separation on Waters ACQUITY UPLC I-Class system.

Figure 2. Plasma Preparation Workflow



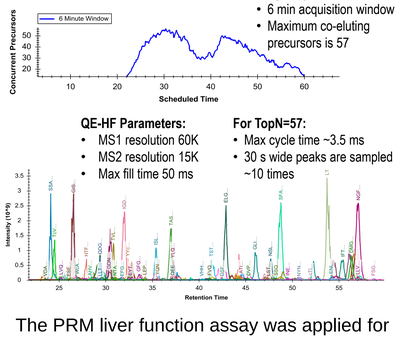
Peptide mapping experiments were carried out for each high pH fraction. Mascot distiller database search was used for peptide sequencing. Greater than 70% of the protein targets of interest from internal and published datasets were identified.

Biomarker List	Reported DILI proteins (Proteins detected by us)
Internal Safety Assessment	48 (41)
Hood publications (human/mouse)	176 (124)
Combined biomarker list	211 (154)

PRM Assay: 231 peptides representing 118 proteins

PRM was performed on a Q-Exactive HF mass spectrometer equipped with a Waters Nano Acquity LC. Retention time standard (iRT) peptides facilitated MS2 scheduling.

Figure 3. Full PRM panel coverage in 2 hr of LC-MS analysis time



The PRM liver function assay was applied for the analysis of plasma from APAP treated (n=1, 3 technical replicates) and ticlopidine treated (n=4) rats. 50 µg of total protein, measured by BCA after depletion, was digested and 0.5 µg was injected on column.

Results

Preliminary Differential Analysis

Figure 4. Log2 Fold Change (p<0.05) in control vs. treated rat plasma

Protein	Peptide	APAP	Ticlopidine
sp|P06866|HPT_RAT	HTFCAGLTK	-3.47	
trj|Q6MG90|Q6MG90_RAT	LSSGNDFVLLR	-2.40	
sp|Q63416|ITIH3_RAT	ATPANLEEAR		-1.18
sp|Q63416|ITIH3_RAT	FAHNVVTTR		-1.15
sp|P04937|FINC_RAT	TFYSCTTEGR		-1.00
sp|Q64240|AMBP_RAT	CIQFIYGGCK		-0.94
sp|Q03626|MUG1_RAT	ISLCHGNPSFSSETK		-0.86
sp|P20059|HEMO_RAT	GGNNLVSGYPK		-0.81
trj|B2RYM3|B2RYM3_RAT	EVAFDVEIPK		-0.47
sp|P07151|B2MG_RAT	TPQIQVYSR		1.04
sp|Q62975|ZPI_RAT	IFSTSADLSELSAVAR		1.15
trj|Q5EBA7|Q5EBA7_RAT	TTDVTQTFAIEK		1.16
sp|P02764|A1AG_RAT	IFAHLIVLK	2.36	
sp|P25093|FAAA_RAT	ASSVVVSGTPIR	4.17	
sp|P07632|SODC_RAT	DGVANVSIEDR	5.11	
trj|G3V6C2|G3V6C2_RAT	FSVDVFEETR	5.12	
sp|P04905|GSTM1_RAT	LYSEFLGK	6.26	
sp|P10760|SAHH_RAT	VADIGLAAWGR	6.58	
trj|D3ZN65|D3ZN65_RAT	GGNASNSCTVLSLLGAR	7.46	
sp|Q63276|BAAT_RAT	LTAVPLSALVDEPVHIR	7.54	
sp|Q6DGG1|ABHEB_RAT	AVAIDLPGLGR	7.58	
sp|P13444|METK1_RAT	SGVLPWLRPDSK	8.93	
sp|P07632|SODC_RAT	VISLSGEHSIIGR	9.50	
sp|P80254|DOPD_RAT	FFPLEPWQIGK	10.97	
trj|G3V6C2|G3V6C2_RAT	YISGFGNECASEDPR	20.19	
sp|Q03336|RGN_RAT	VGVDAPVSSVALR	23.59	

Figure 5. Peak area of VGVDAPSSVALR from RGN in APAP treated rat

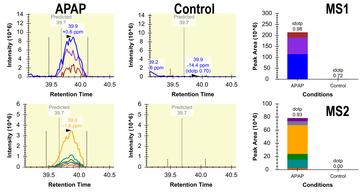
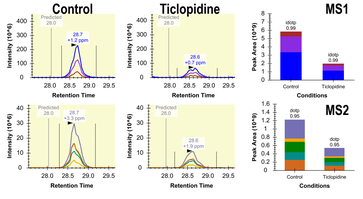


Figure 6. Peak area of ATPANLEEAR from ITIH3 in ticlopidine treated rat



Peptide fold change in treated samples relative to controls was calculated based on MS2 peak areas. Statistically significant ($p<0.05$) values are listed in **Figure 4**.

APAP exposure promoted liver injury, and several peptides were highly elevated. VGVDAPVSSVALR from Regucalcin (RGN) was exclusively detected in APAP plasma (**Figure 5**).

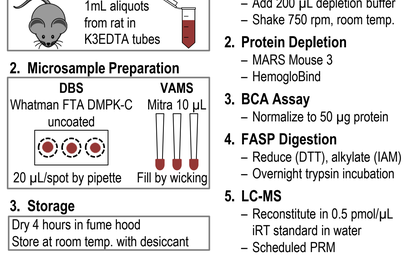
Ticlopidine treatment, which did not cause liver injury in this model, resulted in peptide attenuation, the meaning of which is not clear but could be linked pharmacology. The greatest decrease, ~2 fold, is observed for ATPANLEEAR (**Figure 6**) from Inter-α-trypsin inhibitor heavy chain (ITIH3).

Alternative statistical analysis and internal standards for global normalization are currently being explored to improve the accuracy of the differential analysis.

Applications to Microsampling

The workflow developed for plasma preparation was adapted for DBS and VAMS samples.

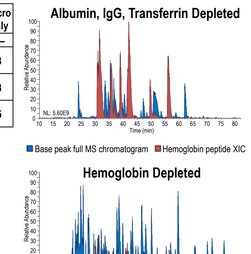
Figure 7. Microsampling Workflow



DILI biomarker panel coverage in DBS and VAMS

Figure 8. Protein depletion evaluation

	Total Protein	Both	Plasma only	Micro only
Plasma	80	—	—	—
MARS VAMS	53	50	30	3
HbBind VAMS	78	70	10	8
HbBind DBS	72	67	13	5



Hemoglobin overwhelms lower abundant biomarker proteins in MARS depleted VAMS samples. After hemoglobin depletion, panel coverage in VAMS and DBS approaches that observed for traditional plasma samples.

Conclusions

1. Sample preparation method has been optimized for the discovery-based and targeted proteomic analysis of plasma
2. Rat DILI biomarker candidate library, containing 154 proteins, was constructed based on in-depth analysis of rat plasma
3. Scheduled PRM targeting 118 proteins in a single LC-MS injection was applied to APAP and ticlopidine (negative control) treated rats and different toxicity patterns were observed
4. Hemoglobin depletion improves DILI panel coverage in DBS and VAMS

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